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Rapid protein uptake and digestion in proximal tubule lysosomes

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Rapid protein uptake and digestion in proximal tubule lysosomes. The aim of the present study was to determine the initial time course for the handling of protein by the proximal tubule cells in rat kidney, using ^{125}I-labeled cytochrome C as a tracer. The uptake of the protein was followed by electron microscope autoradiography in kidneys from rats fixed by perfusion after i.v. injection of cytochrome C. Protein degradation was studied by incubating cortical slices, taken from rats, injected with the same label, and measuring the release of trichloroacetic acid (TCA)-soluble radioactivity from the slices. It was shown by autoradiography that lysosomes in proximal tubule cells start to accumulate labeled cytochrome C within 3 min after injection of the protein, and that the concentration of label in lysosomes increases during the first 30 min after injection, whereas it decreases in endocytic vacuoles. The catabolism of protein as measured in cortical slices began within 13 min after the i.v. injection. Other experiments showed that the accumulation of cytochrome C in the kidneys is very fast. The maximum accumulation, 37% of the injected dose, was reached seven minutes after injection. The results show that protein uptake in proximal tubule cells, transport into lysosomes and the digestion of protein is a more rapid process than previously reported in ultrastructural studies.

Captation rapide et dégradation de protéines dans les lysosomes du tube proximal. Le but de ce travail est de déterminer l'évolution précoce en fonction du temps du comportement des cellules du tube proximal de rein de rat vis-à-vis des protéines en utilisant du cytochrome C marqué par ^{125}I comme traceur. La captation de la protéine a été suivie par autoradiographie en microscopie électronique de reins de rats fixés par perfusion après injection intraveineuse de cytochrome C. La dégradation des protéines a été étudiée par l'incubation de tranches de cortex, prélevées sur des rats ayant reçu le même marqueur, et la mesure de la radioactivité soluble dans l'acide trichloracétique (TCA) contenue dans ces tranches. Les autoradiographies ont montré que les lysosomes des cellules tubulaires proximales commencent à accumuler le cytochrome C marqué dans les 3 min qui suivent l'injection de la protéine et que la concentration du marqueur dans les lysosomes augmente durant les 30 premières minutes qui suivent l'injection, alors qu'elle diminue dans les vacuoles. Le catabolisme dans les tranches commence dans les 13 minutes qui suivent l'injection. D'autres expériences montrent que l'accumulation du cytochrome C dans les reins est très rapide. L'accumulation maximale, 37% de la dose injectée, est atteinte 7 min après l'injection. Ces résultants montrent que la captation de la protéine par les cellules tubulaires proximales, le transport dans les lysosomes et la dégradation est un processus plus rapide que ce qui a été conclu d'études ultrastructurales antérieures.

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Many different proteins are reabsorbed by proximal tubule cells in the kidney, and transported into the lysosomes [1–6], which contain proteolytic enzymes [7]. Although the general steps in this heterophagocytic process have been well established, the time sequence of the uptake and initiation of lysosomal digestion has received little attention. While the authors of the articles cited above find the absorbed protein in lysosomes at varying times from 7 to 60 min after injection, Davidson, Hughes and Barnwell [8] recently reported, on the basis of experiments with isolated lysosomal fractions, that a significant amount of ribonuclease was located in lysosomes by 1 min after i.v. injection. Pullman, Oparil and Carone [9] have reported digestion products of angiotensin II in the urine within 11 min after microinfusion into proximal tubules.

The present investigation was carried out to determine in more detail the initial time sequence for the absorption and intracellular processing of protein in the proximal tubule. Part of the experiments were performed on renal cortical slices as employed previously for the demonstration of protein catabolism [10]. The process of endocytosis, transport into lysosomes and digestion of the protein within lysosomes was studied by the combined use of electron microscope autoradiography and biochemical methods. Cytochrome C, which can be demonstrated by electron microscope histochemistry [11], was used as a label.

The experiments demonstrated a statistically significant accumulation of tracer over lysosomes in proximal tubule cells as early as three minutes after injection of ^{125}I-labeled cytochrome C. It was also demonstrated that renal cortical tissue slices were able to digest a significant amount of ^{125}I-labeled cytochrome C within 13 min after i.v. injection of the protein.

Methods

Animals. Male Wistar rats, 3 to 6 months of age, were used in all experiments.

Iodination of cytochrome C. Cytochrome C from horse heart (Sigma type IIA; mol wt, 12,400) was iodinated with ^{125}I (Amersham, England) using the iodine monochloride method of McFarlane [12] as modified by Izzo et al [13]. The iodination was carried out with 1 mole of iodine monochloride/mole of cytochrome C. The mean iodination efficiency was 53.4% in nine iodinations. Free iodine was removed by repeated dialysis against 0.9% NaCl and subsequent passage through a Sephadex G-25 column. The average concentration of free iodine in the final solutions was 2.5% (range, 1.1 to 5.6) and the mean specific activity of the labeled protein was 0.32 mCi/mg of cytochrome (range, 0.26 to 0.37). The final solutions contained 1.40 mg of cytochrome C/ml (range, 1.12 to 1.61).

Digestion of ^{125}I-labeled cytochrome C by lysosomal enzymes. To determine if cytochrome C was digestible by lysosomal enzymes, lysosomes were isolated from rat renal cortex as described by Maunsbach [14]. In three experiments lysosomes were isolated and disrupted by repeated freezing and thawing. About 0.4 mg of lysosomal protein in 0.5 ml of a solution containing 0.30M sucrose and 20 mM acetate buffer adjusted to pH 4.5 was incubated with 0.1 to 0.5 μl of labeled cytochrome C. The incubations were carried out at either 0 or 37°C. The digestion of the protein was determined by precipitation of nondegraded protein in 10% trichloroacetic acid (TCA) followed by centrifugation and determination of radioactivity in the pellet and supernatant. Determination of the digestion products was performed, in two experiments, by ascending paper chromatography of the incubation media using acetic acid, *N*-butanol, water; 10:78:12. Sodium iodide, monoiodotyrosine (MIT) and diiodotyrosine (DIT) were used as standards. The chromatograms were developed with palladium chloride and ninhydrin. Distribution of radioactivity in the chromatograms was determined by counting 1-cm wide strips of the paper in a γ-counter (Selektronik, Denmark).

Slice experiments. Fifteen rats, anesthetized i.p. with Nembutal, were injected in the femoral vein with 0.2 to 0.5 ml of ^{125}I-labeled cytochrome C, corresponding to 0.28 to 0.70 mg of cytochrome C. At different time intervals after the injection, 3 min (five animals), 7 min (five animals) and 15 min (five animals), the kidneys were perfused retrograde through the aorta with ice-cold incubation medium. During perfusion the incubation medium was kept at 0°C in order to diminish transport and digestion of protein in the proximal tubule cells. Slices were removed from the kidney cortex as soon as the blood was washed out of the kidneys as indicated by their change in color, which usually occurred within 30 sec. The slices were taken with a Stadie–Riggs–type microtome as described by Maude [15]. They were then transferred to a Petri dish kept at 0°C and cut into smaller pieces, which were incubated at either 0 or 37°C. The time interval from start of perfusion to start of incubation never exceeded three minutes. The incubation medium used was that described by Maude [15] with the addition of 1mM MIT [10]. The protein catabolism was measured as TCA-soluble radioactivity in homogenated slices and incubation medium as a percent of total radioactivity. Radioactivity measurements were performed in a γ-counter (Selektronik, Denmark). The digestion products were identified by ascending paper chromatography of the incubation media as described above.

Plasma concentration. In three experiments the plasma concentration of labeled cytochrome C was determined at different time intervals after i.v. injection as TCA-precipitable radioactivity. Blood samples were taken from a catheter placed in the jugular vein with the body temperature of the rats kept at 37°C.

Kidney uptake. In another series of experiments, involving 12 animals, the total TCA-precipitable radioactivity in the kidneys was determined following homogenization of the tissue at different time intervals after i.v. injection of ^{125}I-labeled cytochrome C.

Electron microscope autoradiography and histochemistry. At different time intervals (3, 7, 15 and 30 min) after injection of ^{125}I-labeled cytochrome C into the femoral vein of rats, the kidneys were fixed by retrograde perfusion with glutaraldehyde through the aorta. Two rats were fixed at each time interval. The fixative consisted of 1% glutaraldehyde in a modified Tyrodes' solution, pH 7.2, containing 2.25% dextran 40. The tissue was then further fixed by immersion for one hour in 3% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.2, and then incubated for acid phosphatase demonstration in the Gomori medium as modified by Barka and Anderson [16]. The incubation was carried out at 37°C for 10 to 20 min. The tissue was then postfixed in 1% OsO_4 in veronal buffer (pH 7.2), dehydrated and embedded in epoxy resin (Epon 812 or Vestopal).

In two animals, rat kidney cortex slices were removed seven minutes after injection of labeled cytochrome C after perfusion retrograde through the aorta with ice-cold incubation medium. The slices were incubated *in vitro* (as described above); after different time intervals, samples were fixed by immersion in 3% glutaraldehyde and prepared as described above.

Thin sections were cut on an ultramicrotome

(LKB), stained with lead citrate or left unstained and prepared for autoradiography by the wire loop method as described by Maunsbach [5] using emulsion (Ilford L_4). Sections were exposed for one to three months, developed for 90 sec in developer (Kodak D19) and fixed for three minutes in 20% sodium thiosulfate. The sections were studied in an electron microscope (Jeol 100B or 100C).

Grain distributions were determined over 37 cross-sectioned proximal tubules from *in vivo* perfusion-fixed tissue (22 tubules) and immersion-fixed incubated slices (15 tubules). The tubules were photographed at magnifications of 2600, 3300 or 5000 and enlarged 2.3 times. All values of grain distributions over tissue compartments were derived from tubules originating from two animals for each time assayed. The autographic background was determined for each tubule by counting grains over Epon from the same section (or the formvar film just beside the section) for an area approximately equivalent to that of the tubule. Since in tubules fixed three minutes after injection of labeled protein the concentration of grains over the compartment with the lowest concentration (nuclei) was more than three times the background, and the concentration over the tissue as a whole was more than 33 times the background, the background level, never exceeding 0.005 grains/μ^2, was considered insignificant in the percent analysis of the flow of label in the proximal tubule cells. However, in the analysis of the concentration of grains over proximal tubules fixed by perfusion after three minutes, all the concentrations were corrected for background (see following).

Grain counts over different regions were expressed as a percent of the total number of grains. The location of a grain was considered to be the center of the smallest circle circumscribing the grain. Grains were determined over lumen, endocytic vacuoles, endocytic region (defined as the region within 0.5 μ from endocytic vacuoles, exclusive of other cell organelles over which grains were determined), brush border, lysosomes, L-cytoplasm (the cytoplasm within 0.5 μ from a lysosomal membrane) and cytoplasm (the remaining cell area including the nucleus).

In a special analysis, the concentration of label was determined over the above-mentioned regions and in addition also over nuclei alone. The analysis was performed on 4 tubules fixed by perfusion three minutes after i.v. injection of labeled cytochrome C (the 4 tubules were included in the 22 tubules analyzed by the percent analysis described above). The areas were measured by point counting [17] and the concentration of label over the different regions was determined and corrected for background.

Results

Autoradiographic localization of i.v. injected cytochrome C. Combined electron microscope cytochemistry (acid phosphatase demonstration) and autoradiography showed a rapid flow of label from endocytic vacuoles to lysosomes in proximal tubule cells (Table 1). The number of grains over endocytic vacuoles and the endocytic region decreased from about 72% (52.6 + 19.3%) after 3 min (Fig. 1) to 15% (2.7 + 12.5%) after 30 min. The corresponding values over lysosomes and L-cytoplasm were, after 3 min, 4.9% (3.1 + 1.8%) (Fig. 2) increasing to 57% (38.7 + 18.2%) 30 min after the injection (Fig. 3). As seen in Table 1 there was a gradual decrease in the number of grains over the brush border and a slight increase in the "cytoplasm" (including the nucleus, defined in Methods).

The concentration of grains over the different regions together with nuclei was determined over four tubules fixed by perfusion three minutes after injection of ^{125}I-labeled cytochrome C. Some of these concentrations are listed in Table 2, corrected for background and for the concentration over L cytoplasm as described in the table. The corrected concentration over lysosomes was significantly higher than the concentration over "cytoplasm," ($P < 0.02$, $N = 4$). Both the concentration over lysosomes and that over "cytoplasm" were significantly higher than the background, whereas that over nuclei was not. The concentration over endocytic vacuoles was higher than any of the concentrations listed in Table 2, namely 1.18 grains/μ^2 (see also Table 1).

During *in vitro* incubation of rat kidney cortex

Table 1. Distribution of autoradiographic grains over perfusion-fixed proximal tubules following i.v. injection of ^{125}I-labeled cytochrome C[a]

Time after injection *min*	Lumen	Brush border	Endocytic vacuoles	Endocytic region (0.5 μ)	Lysosomes	L-cytoplasm (0.5 μ)	Cytoplasm	Total grains *N*
3	1.0	15.0	52.6	19.3	3.1	1.8	7.1	1416
7	3.1	12.0	51.4	15.0	4.8	3.6	10.1	714
15	3.4	4.8	24.4	18.5	17.7	11.4	19.8	1197
30	0.9	2.7	12.5	9.0	38.7	18.2	17.9	664

[a] The numbers are given as % of total number of grains.

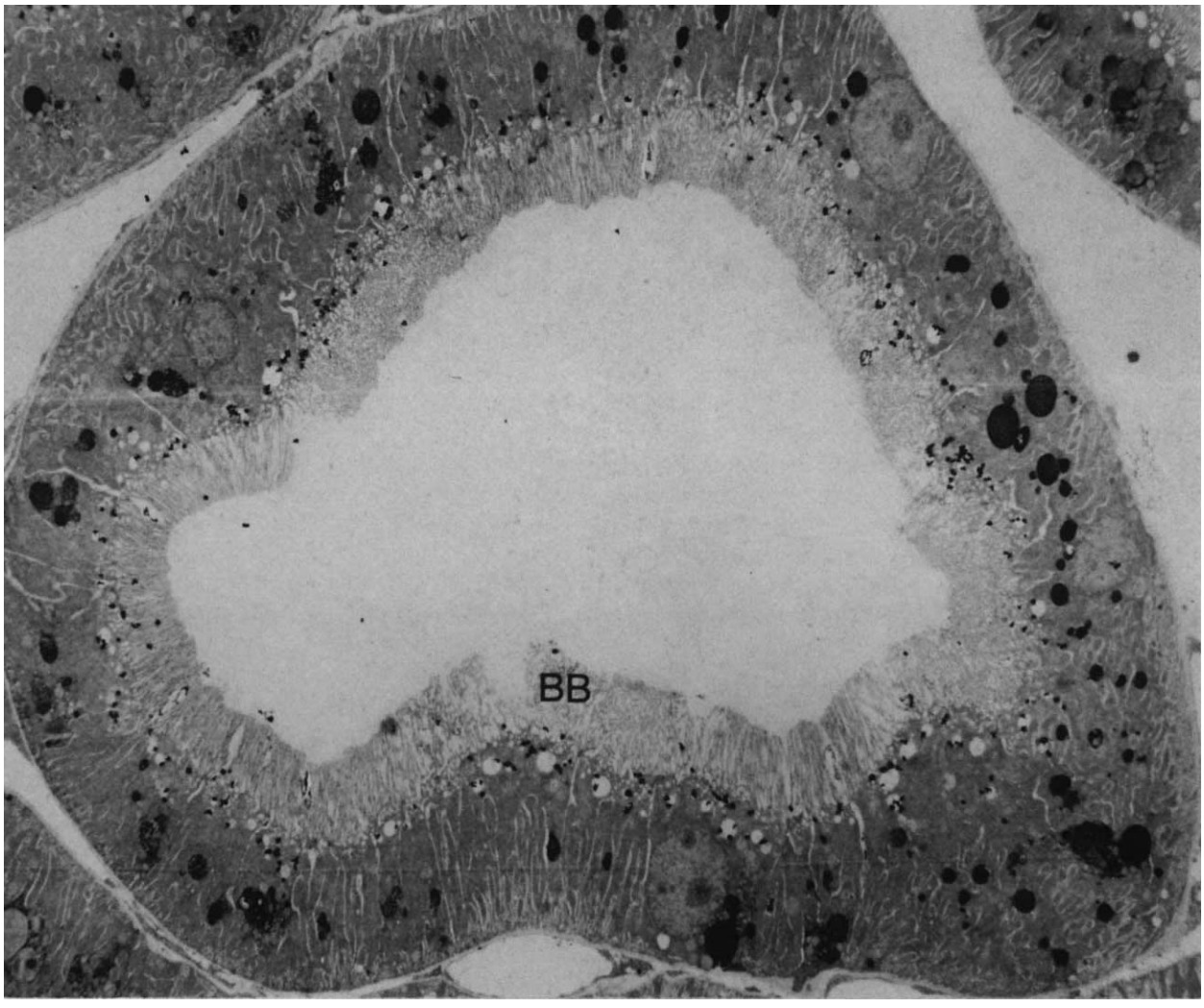

Fig. 1. *Electron microscope autoradiograph of proximal tubule, fixed by perfusion three minutes after i.v. injection of labeled cytochrome C and incubated for acid phosphatase* (×*3,100*). Most of the autoradiographic grains are located in the area just beneath the brush border (BB).

slices removed from the kidneys seven minutes after injection of labeled cytochrome C, there was a flow of label from endocytic vacuoles and the endocytic region to lysosomes and L-cytoplasm in proximal tubule cells (Table 3), but not to the same extent as seen in proximal tubules fixed by perfusion. The number of grains over lysosomes and L-cytoplasm in the slices increased during 30 min of incubation from 17% (13.9 + 3.3%) to 34% (23.8 + 10.4%), whereas the label decreased over endocytic vacuoles and the endocytic region from 68% (57.0 + 11.3%) to 53% (36.2 + 17.0%).

Digestion of labeled cytochrome C in slices. From slice experiments it was shown that the slices removed from cytochrome C-injected animals released TCA-soluble radioactivity during incubation *in vitro*. The experiments also showed that initiation of the release of TCA-soluble radioactivity occurred very rapidly after the onset of incubation. Thus, in slices removed three minutes after the i.v. injection of ^{125}I-labeled cytochrome C the release of TCA-soluble radioactivity had started within ten minutes of incubation at 37°C (Fig. 4). This increase of TCA-soluble radioactivity in slices and incubation medium from the onset (0 minutes), 3.3% ± SEM 0.32, to ten minutes of incubation, 6.8% ± SEM 0.88, was significant ($P < 0.01$, $n = 5$). The rate of digestion increased rapidly up to 30 min of incubation and then at a slower rate up to 120 min. During incubation at 0°C, there was virtually no release of TCA-soluble radioactivity into the medium.

The digestion products from these experiments

were identified by ascending paper chromatography of the incubation medium. As shown in Fig. 5, the increase in TCA-soluble radioactivity was derived mainly from MIT and DIT and this was already evident within ten minutes of incubation.

Experiments in which slices were removed at later time intervals after i.v. injection of labeled cytochrome C showed that the earlier the slices were removed from the kidney the slower was the initial rate of release of TCA-soluble radioactivity (Fig. 6 and Table 4). The results (see Table 4) reveal significant differences between the digestion in slices removed 15 and 3 min after the injection following *in vitro* incubation for 5, 10 or 20 min. Comparison between slices removed after 15 and 7 min shows significant differences after 5 and 10 min of incubation. However, a significant difference between 7 and 3 min (not in the table) was only apparent at 30 min of incubation.

Plasma concentration. The very rapid uptake of labeled cytochrome C in the proximal tubule cells should be compared with the initial very rapid decrease of radioactivity in the peripheral blood (Fig. 7). During the first seven minutes, the concentration decreased to about 20% of that one minute after the i.v. injection. The injected amount of cytochrome C in these experiments was 0.14 mg and the blood concentration one minute after the injection was about 0.003 mg/ml.

Kidney uptake. The uptake of labeled protein in the kidneys (TCA-precipitable radioactivity) was expressed as a percent of total injected cytochrome C at different time intervals after injection in 12 experiments (Fig. 8). As shown in Fig. 8, as much as 25.1% of the injected dose was located in the kidneys after three minutes, the maximum accumulation being 37.0% after seven minutes.

Digestion of labeled cytochrome C with isolated lysosomal enzymes. Lysosomal enzymes obtained from rat renal cortex produced a very rapid degradation of labeled cytochrome C (Fig. 9). During incubation at 37°C about 70% was digested within the first 10 min and about 90% within 60 min. At 0°C there was virtually no digestion of the protein. The digestion products were mainly MIT and DIT as visualized in Fig. 10.

Discussion

The two main results of the present study are: first, the autoradiographic demonstration that some labeled protein becomes located within lysosomes in proximal tubule cells of rat kidney very rapidly (within 3 min, Tables 1 and 2) after i.v. injection and, second, that significant digestion of the labeled protein occurs in renal cortical slices very shortly (within 13 min, Fig. 4) after injection.

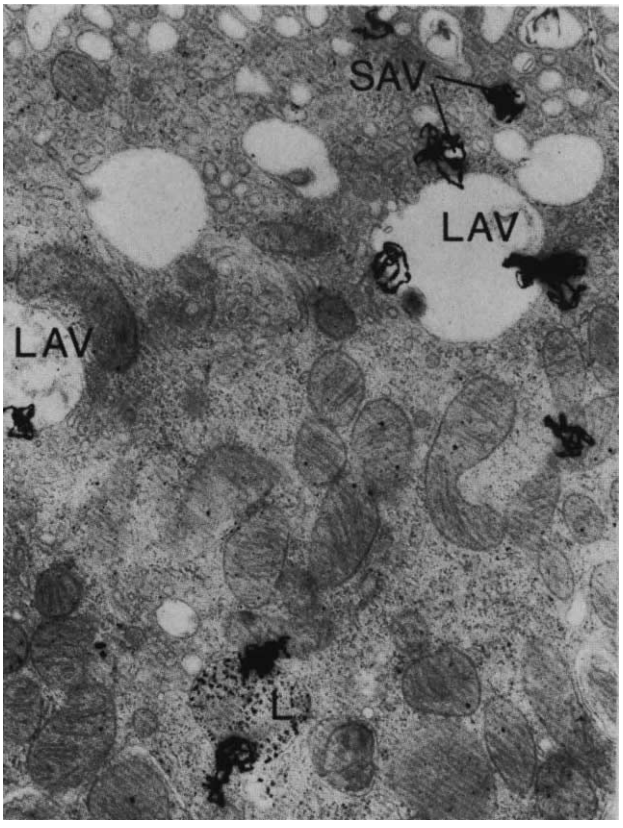

Fig. 2. *Electron microscope autoradiograph from an experiment similar to that illustrated in Fig. 1 (×25,000).* The upper part of the picture shows labeled small (SAV) and large apical vacuoles (LAV). In the lower part a labeled lysosome (L) is observed.

Straus did not observe peroxidase in lysosomes of rat proximal tubule cells before 15 min following i.v. injection of the protein into rats, and he demonstrated that the process of fusion between endocytic vacuoles and lysosomes started between 15 to 20 min after the injection [18]. Using electron microscope histochemistry Graham and Karnovsky [2] observed that peroxidase was located in lysosome-like bodies in proximal tubule cells seven minutes after i.v. injection of the protein into mice, while Maunsbach [5] demonstrated by electron microscope autoradiography that ^{125}I-labeled albumin was located over small apical lysosomes ten minutes after microinfusion into the proximal tubule lumen.

However, the results of the above studies cannot be compared directly to the results of the present study. Peroxidase (mol wt, 40,000) is not filtered to the same extent in the glomeruli as is cytochrome C. Filtration of macromolecules in the glomeruli is not only dependent on their size and molecular weight, but also on the electrical charge of the molecule. Chang et al [19]

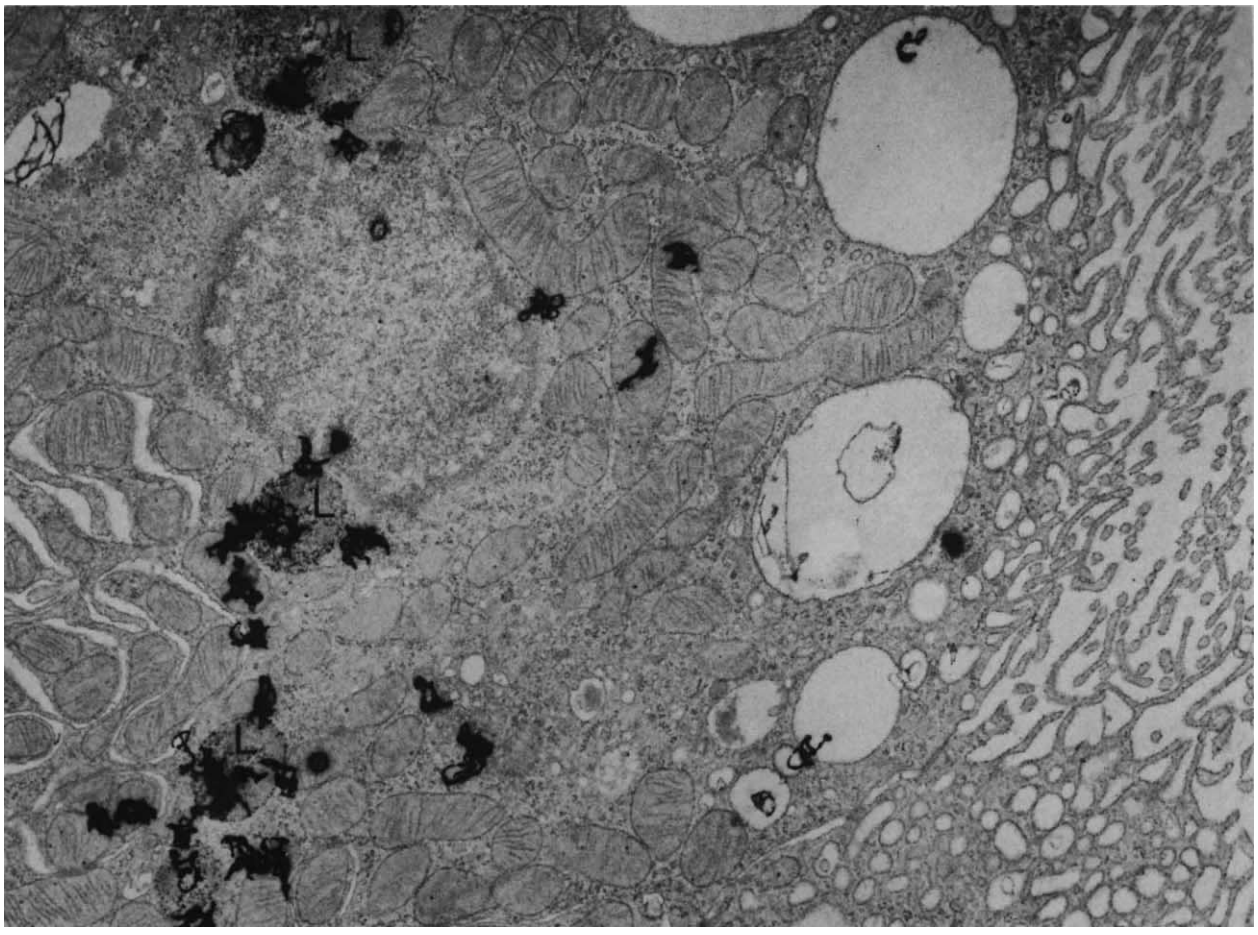

Fig. 3. *Electron microscope autoradiograph from an experiment similar to that illustrated in Fig. 1, but for tissue fixed 30 min after injection of labeled cytochrome C* (×*16,000*). Most of the label is now located over or close to lysosomes (L), whereas only few grains are left in the luminal area of the cell.

compared the fractional clearances for neutral and polyanionic dextrans and showed a reduction in filtration of the polyanionic dextrans. It has also been shown by Farquhar [20] that lysozyme, a cationic protein, binds avidly to the epithelial cell membranes in the glomeruli as well as to the basement membrane. This demonstrates that it is very difficult to compare the renal glomerular handling of macromolecules, unless they are similar with respect to their molecular weight and pI. In the above-mentioned experiments by Maunsbach [5], albumin was infused directly into the proximal tubule lumen, and the uptake and transport into the proximal tubule cells was therefore independent of the filtration in the glomeruli.

The process of binding and uptake of proteins in the proximal tubule cells is probably in some way selective and related to the different charges of proteins. Grinnell, Tobleman and Hackenbrock [21] demonstrated that cationized ferritin binds to anionic sites on baby hamster kidney cells. Thus, cytochrome C, ribonuclease (as used by Davidson, Hughes and

Table 2. The concentration of autoradiographic grains (grains/μ^2) over four tubules fixed by perfusion three minutes after i.v. injection of ^{125}I-labeled cytochrome C[a]

	Lysosomes	L-cytoplasm	Cytoplasm	Nucleus
Observed values	0.080 ± 0.024	0.048 ± 0.015	0.025 ± 0.007	0.015 ± 0.009
Corrected values[b]	0.112 ± 0.032	—	0.025 ± 0.007	0.015 ± 0.009

[a] All concentrations are given ± SEM and corrected for background which was 0.003 ± SEM 0.001.
[b] The area "L-cytoplasm" is added to "cytoplasm" together with the number of grains which corresponds to the concentration over "cytoplasm." The rest of the grains over "L-cytoplasm" are counted as grains from lysosomes.

Table 3. Distribution of autoradiographic grains in slices removed seven minutes after i.v. injection of ^{125}I-labeled cytochrome C[a]

Incubation time *min*	Lumen	Brush border	Endocytic vacuoles	Endocytic region (0.5 μ)	Lysosomes	L-cytoplasm (0.5 μ)	Cytoplasm	Total grains *N*
0	0.5	8.5	57.0	11.3	13.9	3.3	5.5	603
10	0	7.5	47.7	26.4	8.4	3.4	6.7	857
30	0	5.0	36.2	17.0	23.8	10.4	7.6	500

[a] The numbers are given as % of total number of grains.

Barnwell [8]) and lysozyme [10] are cationic proteins, whereas albumin (as used by Maunsbach [5]) is anionic and would therefore probably bind to different sites on the cell membrane. Although from a qualitative point of view no differences have been shown in the uptake by the proximal tubule cells of the different proteins studied, there may, however, be some quantitative differences which remain to be studied.

In the present study the initial accumulation of labeled protein in the lysosomes could be demonstrated by electron microscope autoradiography as early as three minutes after i.v. injection of cytochrome C (Table 1). The number of grains (4.9%) located over lysosomes and L-cytoplasm in proximal tubule cells after three minutes corresponds to 1.2% (4.9% of 25.1%) of the total injected amount of cytochrome C, comparing Table 1 and Fig. 8.

The analysis of the grain distribution indicates that the grains located over lysosomes after three minutes represent a true accumulation of protein. Thus, the concentration of grains over lysosomes was statistically higher not only than the background but also than the "cytoplasm" provided that the lysosomal concentration was corrected for the geometrical spreading of autoradiographic grains (Table 2). Justification for such a correction is the demonstration that the concentration of grains decreases rapidly with increasing distance from the lysosomal membrane in proximal tubule cells from rats injected with ^{125}I-labeled lysozyme [10]. For geometrical reasons spreading of autoradiographic grains can always be expected with grains derived from a labeled source [22].

Davidson et al [8] suggested, on the basis of tissue fractionation experiments on mouse kidney, that about 4% of the total dose of i.v. injected bovine ribonuclease became located in the lysosomal fraction within one minute. The present autoradiographic experiments do not show such a rapid accumulation of protein in the lysosomes. It is conceivable that the difference is related to the use of two different proteins. However, it appears more likely that the discrepancies are related to the different methodological approaches. Thus, while autoradiography relates the absorbed protein to defined cytoplasmic organelles, an unpurified lysosomal frac-

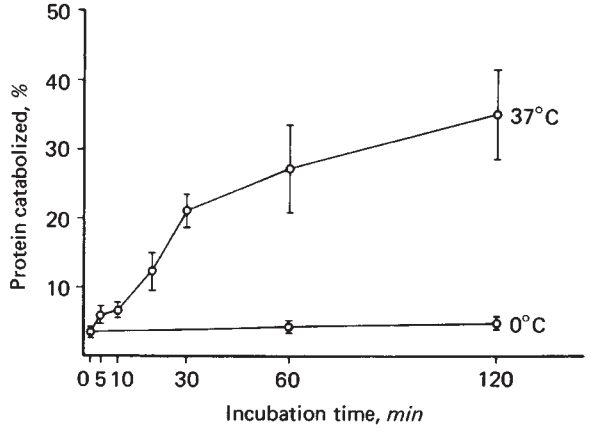

Fig. 4. *Catabolism of ^{125}I-labeled cytochrome C in renal cortical slices removed three minutes after i.v. injection of the protein. Ordinate:* Protein catabolism measured as TCA soluble radioactivity in slices and medium as % of total radioactivity. *Abscissa:* Time in minutes. The curves represent means of five experiments ± SEM.

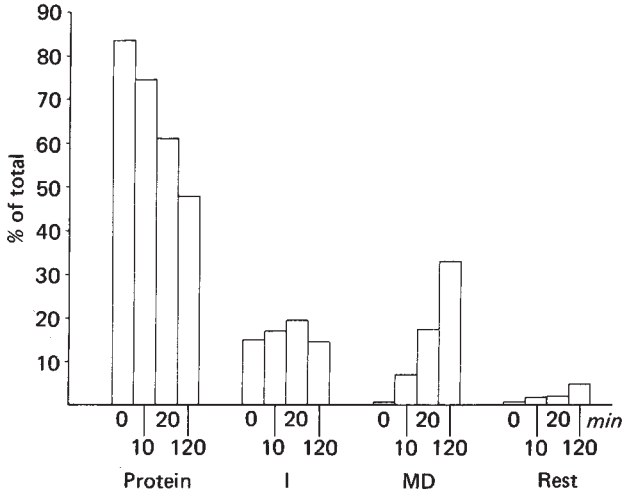

Fig. 5. *Proportions of metabolites in the incubation medium during incubation of renal cortical slices removed three minutes after i.v. injection of ^{125}I-labeled cytochrome C.* The incubation time in minutes is indicated below the chromatogram. Protein, Prot; sodium iodide, I; monoiodotyrosine and diiodotyrosine, MD; and the rest of radioactivity on the chromatogram, Rest. The values are means of two experiments.

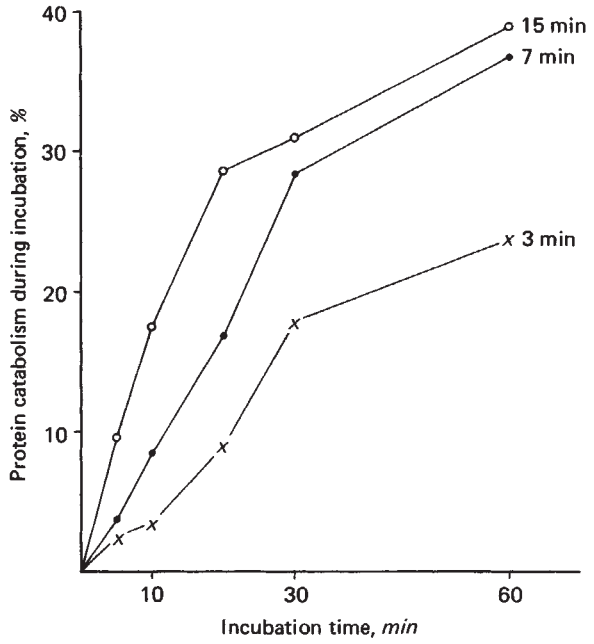

Fig. 6. *Catabolism of ^{125}I-labeled cytochrome C in renal cortical slices removed 3 min (five experiments), 7 min (five experiments) and 15 min (five experiments) after i.v. injection of the labeled protein. Ordinate:* Protein catabolism measured as increase in TCA-soluble radioactivity in slices and medium *during* incubation at 37°C as % of total radioactivity. *Abscissa:* Time in minutes.

tion which has been isolated as described by Davidson et al and which has not been morphologically defined may contain an appreciable mixture of cell organelles, notably contaminating endocytic vacuoles that may carry the bulk of the label. This explanation is supported by the demonstration that only small amounts of labeled β_2-microglobulin could be demonstrated in a highly purified lysosomal fraction which was isolated from rat renal cortex by gradient centrifugation five minutes after i.v. injection of the protein [23].

The present study also demonstrates that a very high amount of the injected dose of ^{125}I-labeled cytochrome C is taken up by the kidneys and digested there. Thus, within 67 min the kidneys had digested at least 14% of the injected dose (after 7 min 37% of the injected dose was located in the kidneys [Fig. 8] and, of that, 36.8% was digested during 60 min of incubation [Fig. 6]). This is probably a minimum value since several factors tend to decrease it. The figure is based partly on *in vitro* conditions and thus the expected continuous reabsorption of labeled cytochrome C into proximal tubule cells after removal of the slice has stopped. It is also noteworthy that cytochrome C in these experiments was not totally digested in the slices. This may be the result of both a decreased transport of labeled protein from endocytic vacuoles to the lysosomes *in vitro* (compare Table 1 and 3), and nonoptimal conditions for protein digestion of cytochrome C was rapid during incubation with isolated lysosomal enzymes (Fig. 9), the conditions for the lysosomal enzymes in a slice incubated *in vitro* for up to one hour may not be optimal. The observation that cells in incubated cortical slices undergo different ultrastructural changes, including cytoplasmic and mitochondrial swelling [10], indicates that metabolic alterations do take place in the cells. These might, for example, result in changes in the pH inside the lysosomes, and thereby interfere with the rate of digestion. However, there is no evidence that digestion of protein in lysosomes in itself is energy-dependent as has been shown by Ehrenreich and Cohn [24], using cultured macrophages.

The decreased transport of protein into the lysosomes may also be due to an altered metabolic activity in the cells in slices. Jamieson and Palade [25] showed that vesicular transport of protein from rough-surfaced endoplasmic reticulum to the con-

Table 4. Statistical significance of differences in digestion of ^{125}I-labeled cytochrome C in renal cortical slices removed at different time intervals after i.v. injection of cytochrome C[a]

		Incubation time, *min*				
		5	10	20	30	60
Removal time[b]	3 min	2.6 ± 0.9	3.5 ± 0.6	9.0 ± 2.4	17.8 ± 2.2	23.8 ± 6.1
Statistical significance		—[c]	—[d]	—[c]	NS	NS
Removal time[b]	15 min	9.7 ± 1.0	17.6 ± 1.7	28.7 ± 4.4	30.9 ± 5.3	39.0 ± 6.1
Statistical significance		—[e]	—[e]	NS	NS	NS
Removal time[b]	7 min	3.7 ± 0.7	8.7 ± 2.4	17.0 ± 4.6	28.3 ± 1.9	36.8 ± 7.8

[a] The numbers represent % protein digested during incubation ± SEM. Each value is the mean of five experiments (except for slices removed after three minutes and incubated for five minutes where there were only three experiments).
[b] Time interval between i.v. injection of ^{125}I-labeled cytochrome C and removal of slices from rat renal cortex.
[c] $P < 0.01$.
[d] $P < 0.001$.
[e] $P < 0.02$.

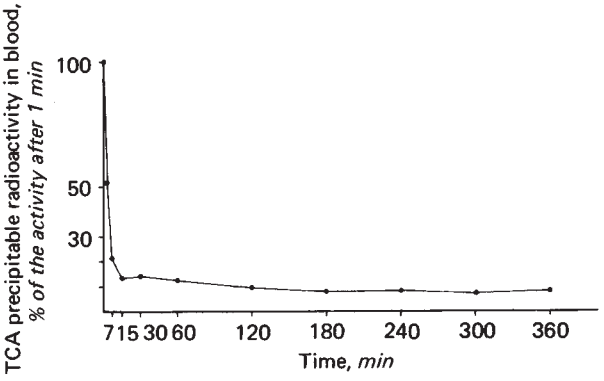

Fig. 7. *Blood concentration of ^{125}I-labeled cytochrome C.* The concentration is expressed as TCA-precipitable radioactivity as % of the TCA-precipitable radioactivity in the blood one minute after i.v. injection of the labeled protein.

densing vacuoles could be blocked by respiratory inhibitors and inhibitors of oxidative phosphorylation, but not by glycolytic inhibitors. The principally similar transport of material from endocytic vacuoles to lysosomes may in some way be influenced by an altered metabolic activity during incubation. However, the transport was only decreased to some extent *in vitro*. Thus, the digestion of the protein probably happens only in the lysosomes, and since 4.9% of the protein (Table 1) was located in the lysosomes at the start of incubation three minutes after the injection and 35% was digested during incubation (Fig. 4), it is probable that at least 30% of the protein initially located in the endocytic vacuoles had been transported into lysosomes during incubation.

It has generally been accepted that protein reabsorbed by proximal tubule cells could be metabolized in the lysosomes, and it has recently been shown more directly [10] by the demonstration that protein undergoing degradation in renal cortical slices is localized in lysosomes in intact proximal tubule cells. This pathway of digestion is supported by the present study. A comparison between Table 1 and Fig. 6 reveals that with increasing time intervals from i.v. injection of ^{125}I-labeled cytochrome C there was a correspondingly higher amount of labeled protein located in the lysosomes, resulting in an initial higher rate of degradation in slices.

Pullman, Oparil and Carone [9] showed that 90%

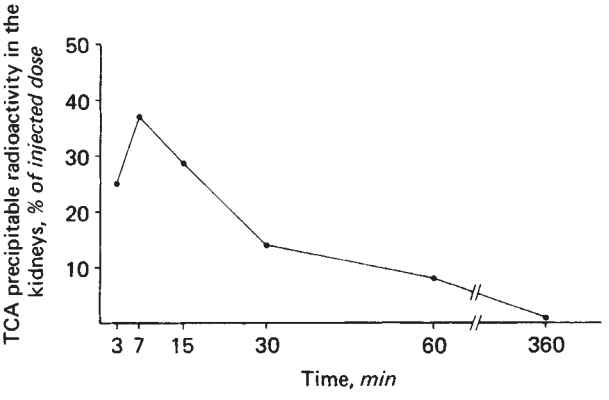

Fig. 8. *Amount of TCA-precipitable radioactivity in the kidneys as % of the i.v. injected dose at different time intervals after the injection of ^{125}I-labeled cytochrome C.* Each *point* represents the mean of two experiments.

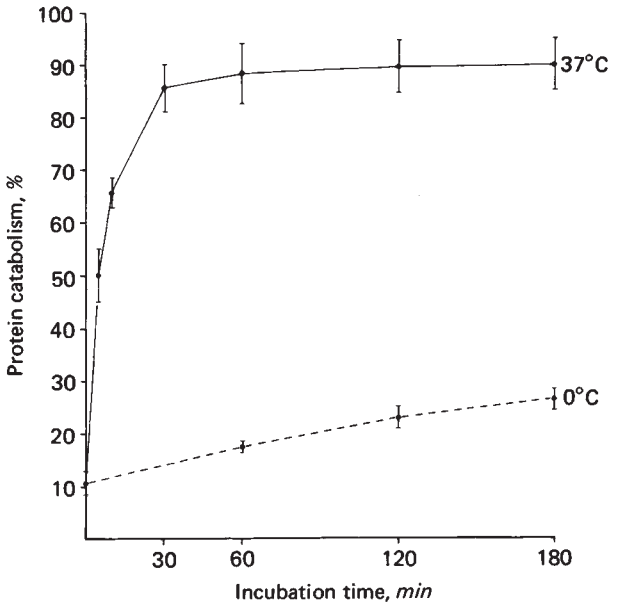

Fig. 9. *Digestion of ^{125}I-labeled cytochrome C by isolated lysosomal enzymes from renal cortex. Ordinate:* TCA-soluble radioactivity as % of total. *Abscissa:* Time in minutes. The values are the mean of three experiments ± SD.

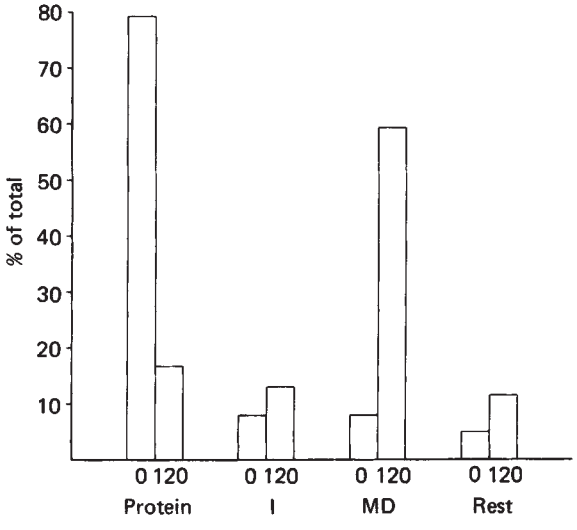

Fig. 10. *Distribution of digestion products, before (0) and after 120 min incubation of ^{125}I-labeled cytochrome C with lysosomal enzymes.* The values are the mean of two experiments. For abbreviations see Fig. 5.

of angiotensin II microinfused into proximal tubules was reabsorbed, and that about 7% of the infused dose was excreted in the urine as digestion products within 11 min after the start of infusion. They proposed that angiotensin II might be degraded by enzymes in the luminal plasma membrane. On the basis of the present data showing protein degradation in slices within 13 min after i.v. injection of labeled cytochrome C, the results of Pullman et al [9] may also be explained by diffusion of digestion products from lysosomal degradation of angiotensin II to the lumen. The possibility cannot yet be excluded, however, that angiotensin II can be degraded by enzymes in the luminal plasma membrane. It must also be considered that angiotensin II is a low molecular weight peptide, which cannot be directly compared to cytochrome C (mol wt, 12,400).

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